Snakin-1, a Peptide from Potato That Is Active Against Plant Pathogens

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A new type of antimicrobial peptide, snakin-1 (SN1), has been isolated from potato tubers and found to be active, at concentrations <10 μM, against bacterial and fungal pathogens from potato and other plant species. The action of SN1 and potato defensin PTH1 was synergistic against the bacterium *Clavibacter michiganensis* subsp. *sepedonicus* and additive against the fungus *Botrytis cinerea*. Snakin-1 causes aggregation of both gram-positive and gram-negative bacteria. The peptide has 63 amino acid residues (M_r 6,922), 12 of which are cysteines, and is unrelated to any previously isolated protein, although it is homologous to amino acid sequences deduced from cloned cDNAs that encode gibberellin-inducible mRNAs and has some sequence motifs in common with kistrin and other hemotoxic snake venoms. A degenerate oligonucleotide probe based on the internal sequence CCEECKC has been used to clone an SN1 cDNA. With the cDNA used as probe, one copy of the *StSN1* gene per haploid genome has been estimated and expression of the gene has been detected in tubers, stems, axillary buds, and young floral buds. Expression levels in petals and carpels from fully developed flowers were much higher than in sepals and stamens. The expression pattern of gene *StSN1* suggests that protein SN1 may be a component of constitutive defense barriers, especially those of storage and reproductive plant organs.

Plants and animals are in close contact with widely diverse bacteria and fungi, but only in rare cases does this association result in the development of disease, substantially because of the existence of defense systems. Although considerable differences exist among different types of organisms with respect to their defense mechanisms, the most notable of which is the lack of an adaptive immune response in plants, recent evidence indicates that both plants and animals share some common features in their defense strategies, namely, the existence of a wide variety of small antimicrobial peptides that are thought to be effector molecules of nonspecific or innate immunity (for reviews see García-Olmedo et al. 1992, 1995; Boman 1995; Hoffmann 1995; Shewry and Lucas 1997; Broekaert et al. 1997). Peptide families identified in animals include both linear and disulfide-folded types (Lehrer et al. 1993; Boman 1995; Hoffmann 1995), whereas only disulfide-containing antimicrobial peptides have been found in plants so far, together with proteins such as glucanases and chitinases, zeamatins, osmotins, and thaumatin-like and ribosome-inactivating proteins (Shewry and Lucas 1997; Broekaert et al. 1997). Thionins, defensins, hevein-like and knottin-like peptides, and the so-called lipid-transfer proteins (LTPs) are among the cysteine-rich plant peptide families that have been shown to be active in vitro against pathogens (García-Olmedo et al. 1992, 1995; Shewry and Lucas 1997; Broekaert et al. 1997). Transgenic overexpression of genes encoding some of these peptides, such as thionins (Carmona et al. 1993; Epple et al. 1997), defensins (Terras et al. 1995), or LTPs (Molina and García-Olmedo 1997), has been shown to confer enhanced tolerance to different pathogens, whereas a marked decrease in virulence has been observed for peptide-sensitive mutants of some pathogens (Titarenko et al. 1997; López-Solanilla et al. 1998).

In plants, a distinction has traditionally been drawn between developmentally regulated (pre-existing) and inducible expression of defense molecules (Bowles 1990), but these alternatives are not mutually exclusive in the case of the plant antimicrobial peptides because they are often encoded by multigene families in which some of the genes are constitutively expressed in certain tissues—typically of storage and reproductive organs—and pathogen-inducible in other parts of the plant, such as leaves (García-Olmedo et al. 1995; Moreno et al. 1994). Furthermore, some genes, as for example those encoding LTP4 in barley, might be expressed at basal levels that produce sufficient protein to reach inhibitory concentrations—which would explain at least some cases of (nonhost) resistance—and still be inducible above basal levels by some pathogens (Molina and García-Olmedo 1993; García-Olmedo et al. 1996; Molina et al. 1996). An external association with the cell wall in peripheral cell layers and in vascular tissues has been often observed for constitutively expressed plant antimicrobial peptides (Molina and García-Olmedo 1993; Shewry and Lucas 1997; Broekaert et al. 1997).

We report here on a novel plant antimicrobial peptide, designated snakin-1 (SN1), that has been isolated from potato

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Nucleotide and/or amino acid sequence data are to be found at GenBank as accession number AF014396 (for *StSN1*).

tubers and is active against potato bacterial and fungal pathogens, as well as against pathogens of other plant species. The amino acid sequence of snakin-1 has some motifs in common with hemotoxic, desintegrin-like snake venoms and is homologous to deduced amino acid sequences corresponding to a family of plant cDNAs of unknown function, some of which are induced by gibberellic acid (GA) in mutants deficient in this hormone (Shi et al. 1992; Herzog et al. 1995; Ben-Nissan and Weiss 1996), whereas one of them has been found to be expressed during lateral root differentiation (Taylor and Scheuring 1994).

RESULTS

Isolation and characterization of snakin-1.

A novel plant antimicrobial peptide, designated snakin-1 (SN1), was isolated from potato tubers in the course of an investigation of possible constitutive defense barriers in this storage organ. A crude cell wall preparation was obtained and extracted with 1.5 M LiCl. This extract was fractionated by RP-HPLC (reverse phase-high pressure liquid chromatography; Fig. 1A) and the fractions were screened for their ability to inhibit in vitro growth of the bacterial potato pathogen *Clavibacter michiganensis* subsp. *sepedonicus* (at 100 μg/ml). The fraction indicated in Figure 1A was found to be homogeneous by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Fig. 1B) and by MALDI-MS (matrix assisted laser desorption ionization mass spectrometry; Fig. 1C). The yield of this protein was 0.3 mmol per kg of fresh weight. The N-terminal amino acid sequence of this peptide was determined up to the 35th residue without any sequence heterogeneity being found (Fig. 1D). A degenerate oligonucleotide probe based on the internal sequence CCEECKC (positions 29 to 35) was used to screen a potato tuber cDNA library constructed in the λ-ZAPII vector. A cDNA clone corresponding to the SN1 peptide sequence was obtained. This cDNA was truncated in the 5′ end, but overlapped with the known N-terminal amino acid sequence (Fig. 1D). Based on the nucleotide sequence of this cDNA, synthetic oligonucleotides were used to clone the 5′ region by the 5′-RACE (rapid amplification cDNA ends) technique (Fig. 1D). The deduced amino acid sequence included a typical signal peptide sequence followed by the known, directly determined N-terminal sequence (Fig. 1D). The relative molecular weight calculated for the SN1 mature protein sequence was 6,922.54, which was in close agreement with the M_r of 6,922.41 directly determined by MALDI-MS (Fig. 1C).

The SN1 amino acid sequence was not related to any previously purified plant protein, although it was homologous to some sequences deduced from cloned plant cDNAs of unknown function, including those designated EST-RC153 from *Ricinus communis* (EMBL accession no. T24153), GAST1 from tomato (Shi et al. 1992), N37340 (Newman et al. 1994), GASA2, and GASA3 from *Arabidopsis thaliana* (Herzog et al. 1995), and Os0951 from rice (Sasaki et al. 1994), as shown in Figure 2. Protein SN1 also had some sequence motifs in common with desintegrin hemotoxic venoms from various snakes, particularly with kistrin, which is a potent platelet aggregation inhibitor and GP IIb-IIIa antagonist from the Malayan pit viper *Agkistrodon rhodostoma* (Adler et al. 1991).

Antipathogenic properties of snakin-1.

The antimicrobial activity of the purified SN1 protein was tested in vitro against the bacterial and fungal species shown in Figure 3A, as well as against *Botrytis cinerea* (EC_{50} = 3 μM). The EC_{50} values for the bacterial species *C. michiganensis* subsp. *sepedonicus* and the fungal species *Fusarium solani* and *B. cinerea,* the three of them pathogens of potato, as well as for the pathogenic fungal species *Bipolaris maydis* and

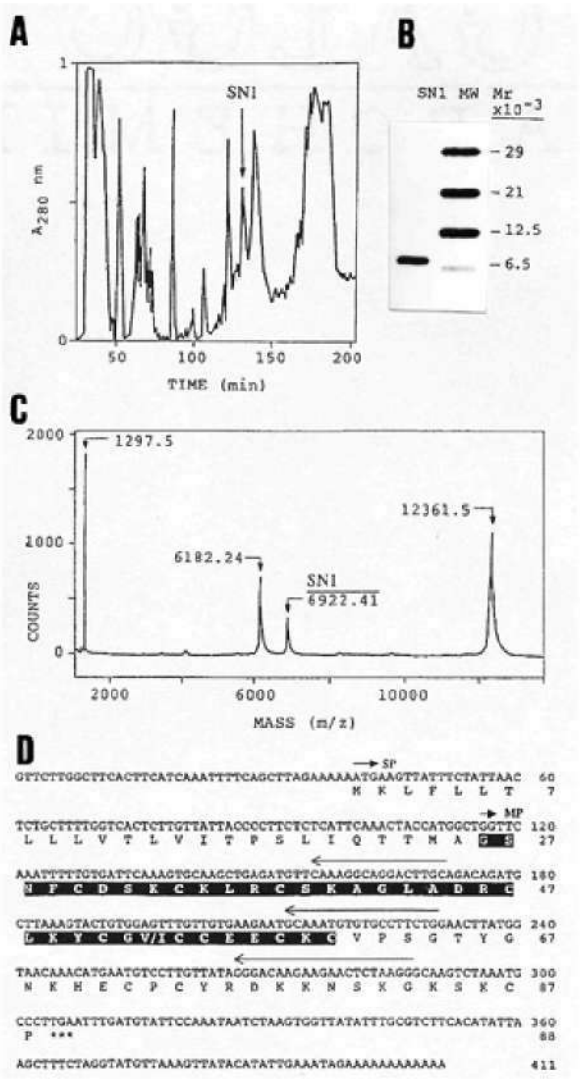

Fig. 1. Characterization of SN1. **A,** Purification of SN1 from a crude cell-wall extract from tubers by RP-HPLC (reverse phase-high-pressure liquid chromatography). **B,** SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) of purified SN1. **C,** MALDI-MS (matrix assisted laser desorption ionization mass spectrometry) determination of SN1 molecular mass. **D,** Amino acid sequence of SN1 and nucleotide sequence of SN1 cDNA. Standards used for SDS-PAGE were bovine lung trypsin inhibitor (M_r 6,500), cytochrome C (M_r 12,5000), soybean trypsin inhibitor (M_r 21,000), and carbonic anydrase (M_r 29,000). MALDI standards were angiotensin I (MH; M_r 1,297.5), and cytochrome C (MH, M_r 1,2361.5; M2H, M_r 6182.2). Shaded amino acid sequence was obtained by direct N-terminal Edman degradation of the protein; the rest of the amino acid sequence was deduced from the nucleotide sequence. The three oligonucleotides used for 5′- RACE (rapid amplification cDNA ends) are indicated by arrows. The 5′-RACE cloned fragment corresponds to nucleotides 1 to 156.

Colletotrichum lagenarium, ranged from 1 to 10 μM, whereas the bacterial potato pathogen *Ralstonia solanacearum* and the fungal species *Aspergillus flavus* were not sensitive to SN1 at the concentrations tested. In contrast, *R. solanacearum* was very sensitive to the PTH1 defensin from potato, whereas the fungus *F. solani,* which was the most sensitive to SN1, was the least sensitive to the defensin (Fig. 3A). A synergistic effect of SN1 with PTH1 was observed against *C. michiganensis,* whereas the combined effect of the two proteins against *B. cinerea* was merely additive (Fig. 3B).

An interesting property of SN1 was its ability to aggregate bacteria, as exemplified in Figure 4. Aggregation occurred very rapidly under conditions in which the α-thionin from wheat used as control had no effect, and affected both gram-positive and gram-negative bacteria (not shown). However, antimicrobial activity of SN1 was not correlated with its capacity to aggregate bacteria.

Expression of gene *StSN1* in the potato plant.

With the cloned SN1 cDNA used as probe, the number of copies of the corresponding gene and its expression pattern were investigated. Southern blot hybridization patterns were compatible with the presence of one or two copies of the *StSN1* gene per genome (Fig. 5). Total RNAs from different parts of the potato plant were analyzed by Northern (RNA) blot and expression of the gene was detected in tubers, stems, axillary buds, and young floral buds, as well as in sepals, petals, stamens, and carpels from fully developed flowers (Fig. 6). Steady-state mRNA levels were particularly high in axillary and floral buds, and in fully developed petals. Expression was not detected in roots, stolons, or leaves. The distribution of *StSN1* mRNA was further investigated by in situ hybridization (Fig. 7). In the flower bud before anthesis (Fig. 7A, B), the strongest signal is detected in the style, in the region of the companion cells of the transmission tissue, and in ovules. The SN1 mRNA is also abundant in the epidermal and subepidermal layers of the young stem and perhaps at low concentration in the vascular bundles, although the strong natural fluorescence from lignified regions interferes with perception of the hybridization signal (Fig. 7C, D). In a section of an incipient tuber (Fig. 7E, F), the signal is absent from the peridermal and root cap regions and is detected in a region corresponding to root apex, beneath root cap and periderm. In the vegetative shoot apex, the signal is absent from the meristem and the young leaves, whereas it is strong in the stem region, especially in epidermis, subepidermis, and cortex (not shown).

Lack of response of gene *StSN1* to abiotic and biotic stimuli.

Young potato leaves, in which developmentally regulated expression of the *StSN1* gene had not been detected, were chosen to test whether this gene was induced in response to abiotic or biotic stimuli. Leaves were sprayed with solutions of hormones that have been implicated in plant-pathogen interactions, such as methyl jasmonate (50 μM), ethylene (ethephon 0.03 g/liter), and abscisic acid (100 μM), inducers of systemic acquired resistance, such as salicylic acid (5 mM) and isonicotinic acid (0.6 mM), and hormones that have been shown to affect steady-state levels of mRNAs encoding some SN1 homologues in other plant species (Shi et al. 1992; Herzog et al. 1995; Ben-Nissan and Weiss 1996), such as indolacetic acid (20 μM) and GA (100 μM). In none of these treatments was induction of the SN1 mRNA observed.

Leaves were also inoculated with an incompatible bacterial pathogen, *P. syringae* pv. *tomato* DC3000 (20 μl of 1×10^8 CFU per ml), and a compatible fungal pathogen, *B. cinerea* (10 μl of 10^6 spores per ml), and no response of the *StSN1* gene during the first 72 h after inoculation was observed in either case (not shown). The bacteria produced necrotic lesions within 12 h that did not extend beyond the inoculated area after several days, whereas the fungi caused necrotic and chlorotic lesions beyond the inoculated area that eventually covered the whole leaf. In both cases, a systemic response was observed for the defensin gene *StPTH1,* which was used as control (not shown).

DISCUSSION

Protein SN1 represents a novel family of cysteine-rich plant antimicrobial peptides that seem to be present in both monocotyledonous and dicotyledonous species, as judged from the occurrence of homologous sequences deduced from previously described cDNA nucleotide sequences. Peptides of this family would have six potential disulfide bridges, whereas previously described families of antibiotic peptides have two to four disulfide bridges (Shewry and Lucas 1997; Broekaert

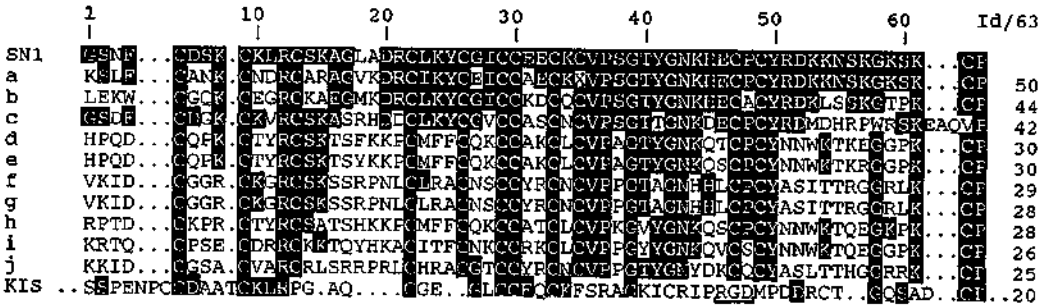

Fig. 2. Alignment of the SN1 amino acid sequence with those deduced from previously reported cDNA nucleotide sequences and that from the hemotoxic snake venom kistrin. Sequences are (a) RC153 from *Ricinus communis* (EMBL accession no. T24153); (b) N37340 from *Arabidopsis thaliana* (Newman et al. 1994); (c) Os0951 from rice (Sasaki et al. 1994); (d) PHGIP1 from petunia (Ben-Nissan and Weiss 1996); (e) GAST1 from tomato (Shi et al. 1992); (f,g,i,j) GASA2,3,4,1 from *A. thaliana* (Herzog et al. 1995); (h) RSI1 from tomato (Taylor and Scheuring 1994); and (KIS) kistrin from the Malayan pit viper (Adler et al. 1991). Identical residues are shaded and the numbers of identical residues (id/63) are indicated. The underlined RGD motif of kistrin is essential for desintegrin action.

et al. 1997). Residues at positions 12 (R), 36-37 (VP), 39 (G), 42-43 (GN), 50 (Y), 57 (G), 61 (K), and 63 (P), as well as all the cysteines are highly conserved in the aligned members of this family. Similarity of SN1 to the snake venom kistrin occurs mainly in two regions, motifs CKLR (residues 9 to 12) and GXCCEXCKXXXXG (residues 27 to 39), but does not include the RGD residues of kistrin (corresponding to positions 46 to 48 of SN1) that are responsible for desintegrin action (Adler et al. 1991). In addition, four of the cysteines from each molecule are not at matching positions. Thus, it is likely that the motifs that are shared by snakin and kistrin might confer some properties related to their biological activities other than the inhibitory or toxic activity itself. The protein is highly basic and has a short, central hydrophobic stretch (residues 25 to 30), which is flanked by highly polar, long N-terminal and C-terminal domains. There are no obvious amphipathic helices in the structure. The presence of a signal peptide in SN1 is congruent with its location in the cell wall.

Only one copy of the *StSN1* gene has been detected in potato by Southern blot, but it is likely that more members of this gene family are present in this species, based on the available data from *A. thaliana,* where at least six widely divergent members of this family have been identified (Herzog et al. 1995; Aubert et al. 1998).

SN1 is active against both bacterial and fungal species, including pathogens from potato, the species from which it has been isolated. No contaminant peptides have been detected in the purified SN1 preparation, using three different criteria, including MALDI-MS analysis, which is very sensitive, so it is unlikely that the observed antimicrobial activity could be due to an impurity. The activity spectrum of SN1 seems to be quite distinct from that of the PTH1 defensin that has also been isolated from potato tubers, which means that the simultaneous presence in the same tissues of the two types of peptides would make the pre-existing inhibitor barrier against

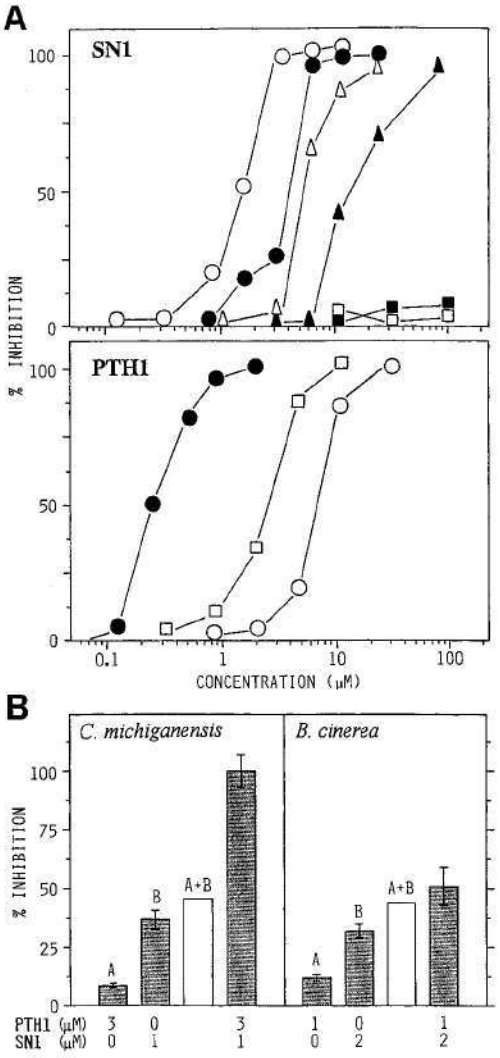

Fig. 3. In vitro antimicrobial activity of SN1. **A,** Inhibition curves of the following pathogens: bacterial species *Clavibacter michiganensis* subsp. *sepedonicus* (open circles), and *Ralstonia solanacearum* (open squares); fungal species *Fusarium solani* (solid circles), *Bipolaris maydis* (open triangles), *Colletotrichum lagenarium* (solid triangles), and *Aspergillus flavus* (solid squares). Inhibition caused by potato defensin PTH1 has been added for comparison. **B,** Synergistic and additive antimicrobial effects of combinations of SN1 and PTH1 against *C. michiganensis* and *B. cinerea.* Open bars represent the sum of the individual effects.

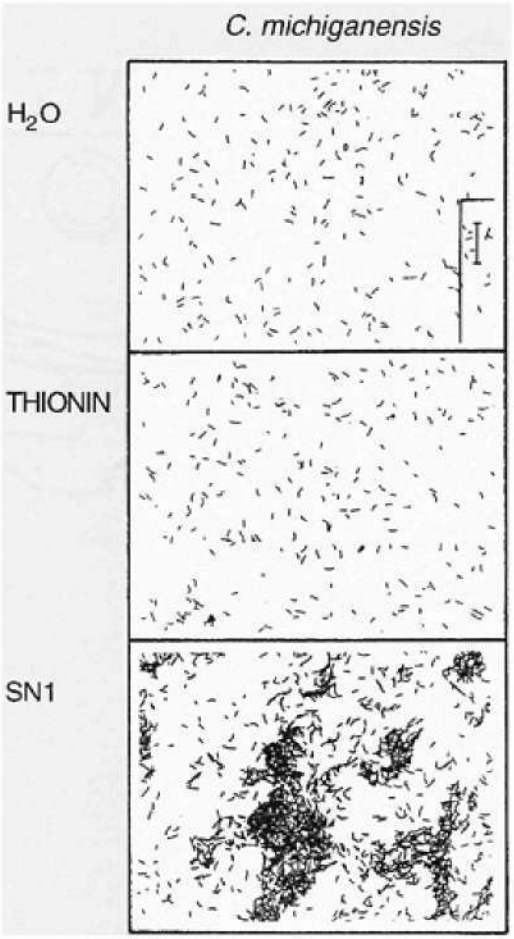

Fig. 4. Bacterial aggregation caused by SN1. Addition of SN1 to a concentration of 10 μM caused immediate aggregation of bacteria. A 5-μl bacterial suspension (10^5 CFU per ml) was dropped in a microscope slide and then 5 μl of a 20 μM solution of the SN1 protein was added. A photograph was taken inmediately under the microscope. The same concentration of thionin was used as control. Bar = 20 μm.

pathogens more polyvalent. The observed synergistic antimicrobial effect of combinations of SN1 and PTH1 is also in line with this hypothesis. No information is yet available concerning the mechanism of action of SN1, except that, in contrast to other plant antibiotic peptides tested, it does not mediate aggregation or leakage of artificial liposomes under low or high salt conditions (Caaveiro et al. 1997). The rapid aggregation of SN1-treated gram-positive and gram-negative bacteria does not seem to be related to SN1 toxicity in vitro because, for example, *R. solanacearum* was not inhibited at SN1 concentrations that did cause aggregation. However, aggregation might play a role in vivo through the control of pathogen migration to uninfected areas. Whether the aggregation mechanism consists in the formation of cationic bridges between the negatively charged bacteria or in the simple neutralization of their surface charges remains to be investigated. Thanatin, an insect defense peptide with sequence homology to some frog skin antimicrobial peptides, has also been reported to aggregate bacterial cells, but in that case the possibility of linking cells through cationic bridges seems less likely because the peptide is only 21 residues long (Felhbaum et al. 1996).

Developmental expression of the *StSN1* gene is consistent with a role of the SN1 peptide as part of pre-existing defense barriers. Preferential expression in storage and reproductive organs is a frequent feature of plant constitutive antibiotic peptides (Molina and García-Olmedo 1993; Moreno et al. 1994; Shewry and Lucas 1997; Broekaert et al. 1997). Thus, the *StPTH1* gene from potato, encoding defensin PTH1, is also preferentially expressed in tubers and flowers, although there are some differences in its overall expression pattern with respect to gene *StSN1* (Moreno et al. 1994). Expression patterns of *StSN1* homologues from *A. thaliana* are quite diversified: some of them are seed specific while others are more widely expressed in an overlapping fashion in different parts of the plant (Herzog et al. 1995; Aubert et al. 1998). Thus, it is likely that other members of the SN1 family will be found in those parts of the potato plant, such as roots, stolons, and leaves, where expression of gene *StSN1* has not been detected. Pathogens such as *C. michiganensis* subsp. *sepedonicus* and *B. cinerea,* which infect tubers, and *F. solani,* which infects stems and tubers, might be affected in vivo by SN1, whereas leaf-specific pathogens could be affected by other members of this peptide family that are likely to be expressed in leaves, as judged from the *A. thaliana* data (Herzog et al. 1995; Aubert et al. 1998).

The *StSN1* gene did not respond to abiotic and biotic stimuli, including GA. This does not exclude the possibility that other members of this family in potato might respond to some of these stimuli. Expression of the gene encoding GAST1 from tomato has been shown to be clearly enhanced by GA in GA-deficient mutants (Shi et al. 1992), and a similar though weaker effect has been observed for some GAST1 homologues in *A. thaliana* (Herzog et al. 1995; Aubert et al. 1998), but this behavior, which does not define a function, is not common to all genes of this family. A defense role is not in-

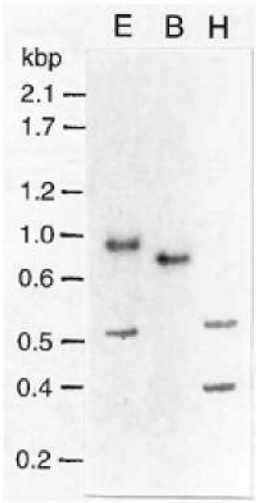

Fig. 5. Southern blot analysis of gene *StSN1*. Genomic DNA (5 µg) was digested with the *Eco*RI (E), *Bam*HI (B), or *Hin*dIII (H) restriction endonucleases. The ^{32}P-labeled SN1 cDNA was used as probe.

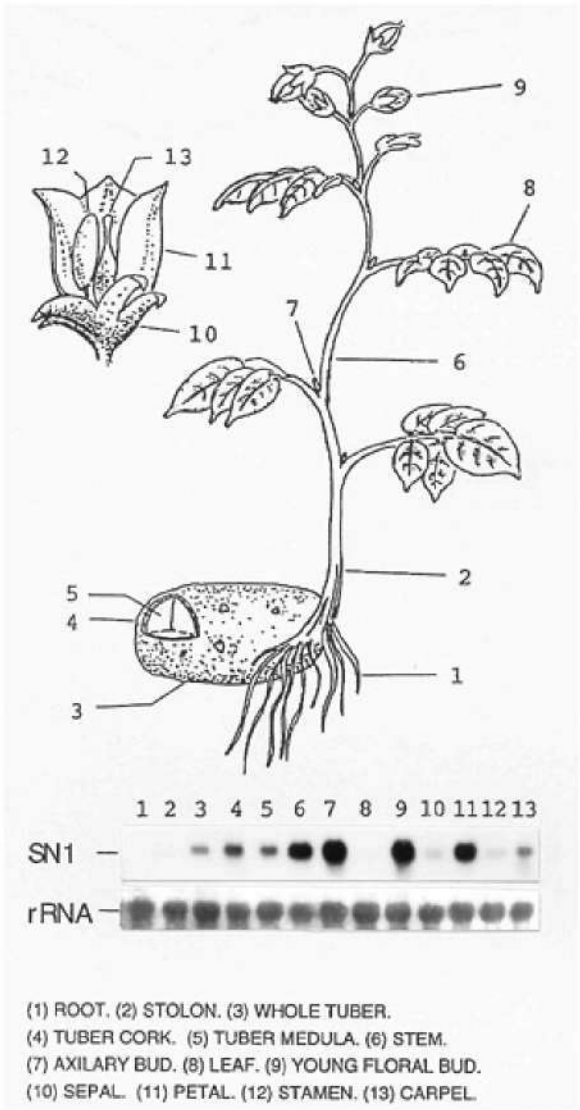

Fig. 6. Expression of gene *StSN1* in potato. Nothern (RNA) blot analysis of total RNAs (5 µg) from the indicated parts of the plant, with the ^{32}P-labeled SN1 cDNA used as probe.

compatible with other possible functions in the plant for all or some members of this protein family. The hypothesis of a defense role for SN1 is also supported by the observation of decreased virulence in potato tubers of SN1-sensitive mutants of the bacterial pathogen *Erwinia chrysanthemi* (Lopez-Solanilla et al. 1998). Antibiotic properties of SN1 analogues remain to be investigated.

MATERIALS AND METHODS

Biological materials.

Solanum tuberosum cv. Desireé was cultivated at 60% humidity and 20°C, with a photoperiod of 18 h light/6 h dark. Bacterial pathogens *C. michiganensis* subsp. *sepedonicus,* strain C5, and R. *solanacearum,* strain P2, as well as the fun-

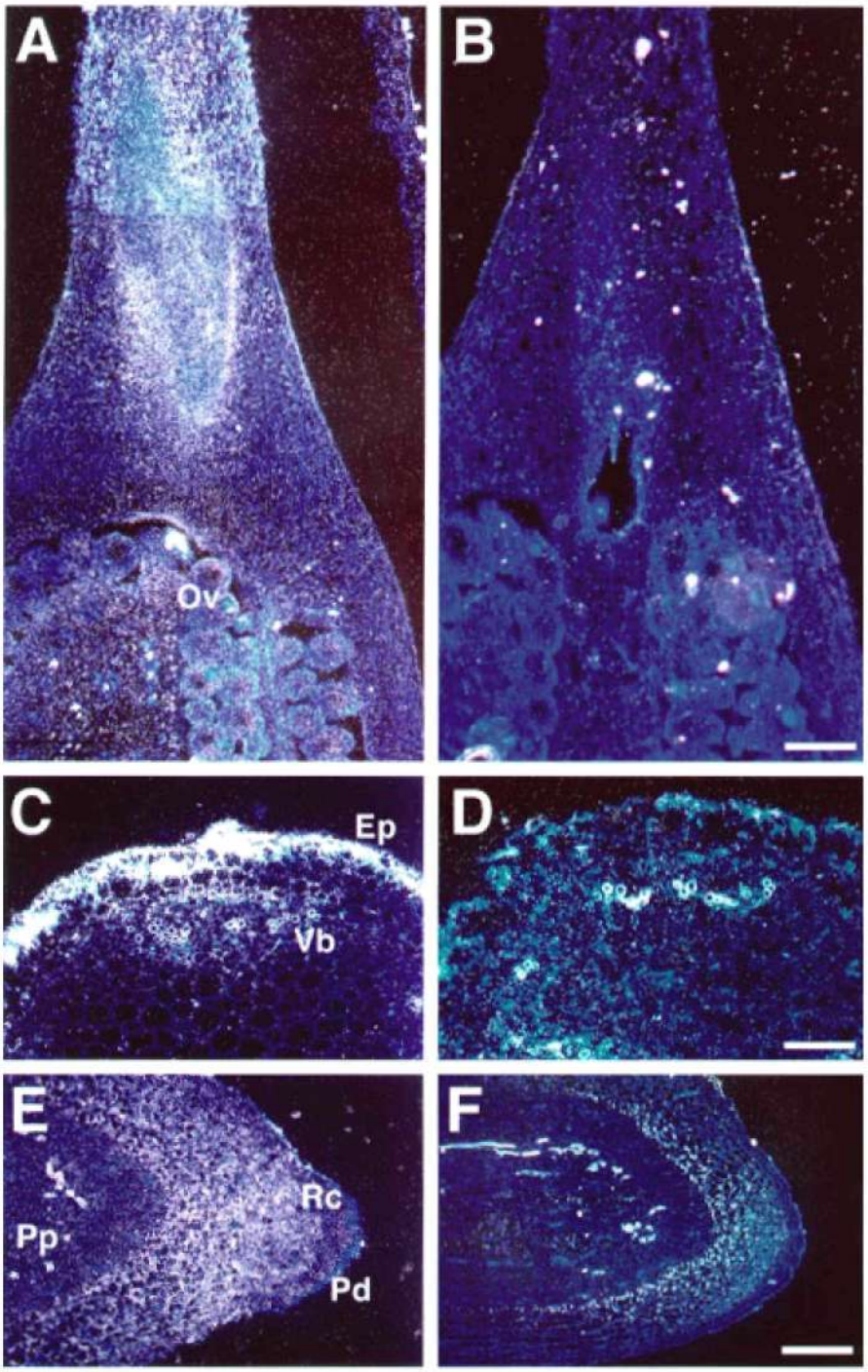

Fig. 7. In situ hybridization with SN1 antisense. **A and B,** Section of a pistil from a potato flower before anthesis; position of ovules (Ov) is indicated. **C and D,** Section of a young stem; epidermis (Ep) and vascular bundle (Vb) are indicated. **E and F,** Section of an incipient tuber; pith parenchyma (Pp), root cap (Rc), and peridermis (Pd) are indicated. Negative hybridization controls (**B, D,** and **F**). Bar = 200 µm.

gal pathogens *B. cinerea*, strain 1, and *F. solani*, strain 1, were from the ETSIA collection (Madrid). The bacterium *P. syringae* pv. tomato DC3000, and the fungal species *A. flavus*, *B. maydis*, *F. culmorum*, and *C. lagenarium* were from the Novartis Corp. collection (Research Triangle Park, NC).

Purification and characterization of the protein.

Frozen tuber material (20 g) was ground to powder in liquid nitrogen with a mortar and pestle, and extracted once with 80 ml of buffer (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5) and twice with 80 ml of H_2O. The resulting pellet was then extracted with 50 ml of 1.5 M LiCl at 4°C for 1 h, and the extract dialyzed against 5 liters of H_2O, with a Spectra/Por 6 (MWCO:3000) membrane (Spectra, Laguna Hills, CA), and freeze dried. The extract was subjected to RP-HPLC on an Ultrapore C3 column (1 × 25 cm; 5 μm particle; 300 A pore; Beckman, San Ramón, CA), with an H_2O/2-propanol (0.1% trifluoroacetic acid) gradient (0 to 40%, 240 min), at 0.5 ml/min. Fractions were collected by hand and freeze dried. The proteins were subjected to SDS-PAGE in preformed gradient gels (4 to 20%; BioRad, Hercules, CA) according to the manufacturer's instructions. MALDI-MS was performed with the Voyager Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA). Amino acid sequencing was carried out by automated Edman degradation of the intact protein.

Characterization of cDNA; Northern and Southern blots.

A tuber cDNA library (cv. Jaerla; λ-ZAPII; Stratagene, La Jolla, CA) was blotted on nylon membranes (Hybond N; Amersham, Rainham, UK) and screened with a degenerate oligonucleotide probe corresponding to the internal amino acid sequence CCEECKC. Hybridization was done at 58°C under previously described conditions (Church and Gilbert 1984). The selected cDNA clone was sequenced by the dideoxy chain-termination method (Sanger et al. 1977). The 5′ region of the SN1 cDNA was cloned with the 5′/3′-RACE Kit from Boehringer (Mannheim, Germany) with total RNA extracted from potato tubers and the primers indicated in Figure 1D. SN1 cDNA was random-labeled with ^{32}P-dATP following standard procedures (Sambrook et al. 1989) and used as a probe. RNAs were purified from frozen tissues by phenol/chloroform extraction, followed by precipitation with 3 M LiCl (Lagrimini et al. 1987), and subjected to electrophoresis on 5% formaldehyde/agarose gels. The gels were blotted to Hybond N membranes (Amersham). Equal sample loads were checked by hybridizing with a ribosomal cDNA probe. Radioactive signals in Northern blot experiments were quantified by densitometry of three independent filters. Southern blots were done as described (Sambrook et al. 1989). Hybridization and washing of Northern and Southern blots were carried out at 65°C according to Church and Gilbert (1984).

In situ hybridization.

Previously described procedures were used (Huijser et al. 1992). The tissue was fixed in a mixture of 2% formaldehyde, 5% acetic acid, and 60% ethanol for 48 h at 4°C. Sections of paraffin-embedded tissue were hybridized with a mix containing 1 × 10^7 cpm per ml of ^{35}S-labeled antisense or sense RNA probes that were synthesized from the 255-bp insert of the SN1 clone cloned in pBluescript (Stratagene, La Jolla, CA) according to the protocol supplied by Ambion (Austin, TX). For autoradiography, the hybridized slides were dipped in Kodak NTB2 emulsion and stored for 4 weeks at 4°C before development. Silver grains were observed with dark-field illumination, and the underlying tissue, stained with Calcofluor, was examined by UV epifluorescence.

Pathogen inhibition tests and plant inoculations.

For inhibition tests, 50-μl aliquots of bacterial suspension in nutrient broth (Oxoid, Basingstoke, UK) were mixed with different amounts of the protein dissolved in 100 μl of sterile water, so that the final bacterial concentration was 10^4 CFU per ml, in sterile microtiter plates. After 16 to 24 h of incubation at 28°C, growth was recorded by measuring absorbance at 490 nm in an ELISA (enzyme-linked immunosorbent assay) plate reader. Fungal spores were collected from 8-day-old cultures grown at 25°C on potato dextrose agar plates (Difco, Detroit, MI) and stored at –80°C in 20% glycerol. Spore suspensions (10^4 per ml) were incubated in microtiter plates with the indicated amounts of added proteins (50 μl of protein solution + 25 μl of potato dextrose broth; Difco) at 25°C for 26 to 44 h and growth was recorded as above. The potato PTH1 defensin included in the pathogen inhibition experiments was prepared as previously described (Moreno et al. 1994), and the α-thionin used as control in the aggregation experiment was the gift of P. Rodriguez-Palenzuela (Madrid).

P. syringae pv. *tomato* DC3000 was cultured overnight in nutrient broth (Oxoid), resuspended in 10 mM $MgCl_2$ to 10^8 CFU per ml, and inoculated at multiple points in the leaves with a blunt-ended syringe (20 μl per point). Mock inoculation with 10 mM $MgCl_2$ was carried out in the same manner. Leaves were collected at 0, 12, 24, 48, and 96 h and frozen in liquid nitrogen. *B. cinerea* was cultured on solid potato dextrose agar medium (Difco) at 25°C and spores were collected in sterile water. Spores (10 μl of 1 × 10^6 per ml) were inoculated as above. Leaf samples were collected at 0, 24, 48, and 72 h after inoculation. Mock inoculations were done with sterile water.

External treatments.

Plants grown as described above were used throughout these treatments. Treatments with methyl jasmonate (50 μM; Bedoukian Research, Danbury, CT), abscisic acid (100 μM; Sigma, St. Louis, MO), sodium salicylate (5 mM; Sigma), ethylene (ethephon 0.03 g/liter), 2,6-dichloro-isonicotinic acid (0.6 mM; Novartis Corp., Basel, Switzerland), indolacetic acid (20 μM; Sigma) and GA (100 μM; Sigma) were done by spraying 25-day-old plants (20 plants per treatment). Leaves were collected at 12, 30, and 84 h after treatment.

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